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Plaque Assay of Nuclear Polyhedrosis Viruses in Cell Culture

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The nuclear polyhedrosis virus of *Autographa californica* has been titrated in *Spodoptera frugiperda* cells by the plaque method, using a solid overlay which does not require either the use of modified culture medium or expensive purified agarose or the addition of culture medium as a liquid layer above the solid agarose. This assay is more sensitive than that using a viscous methyl cellulose overlay but less sensitive than the end-point dilution technique. Neither *Trichoplusia ni* nor *Bombyx mori* cells were satisfactory as indicators for the assay as described, since they failed to form a stable monolayer. *Manduca sexta* cells could be utilized for assay of *A. californica* nuclear polyhedrosis virus, but the sensitivity was lower than with *S. frugiperda* cells.

The plaque assay technique was first applied to baculoviruses by Hink and Vail (9), who utilized a methyl cellulose overlay procedure. Subsequent techniques utilizing a solid overlay were developed by Wood (12; Proc. First Int. Colloq. Invertebr. Pathol. and IXth Annu. Meet. Soc. Invertebr. Pathol., 1976, abstract, p. 401-402), Knudson (Proc. First Int. Colloq. Invertebr. Pathol. and IXth Annu. Meet. Soc. Invertebr. Pathol., 1976, abstract, p. 104-107), and Brown and Faulkner (2). The technique reported here, which does not require the use of modified culture medium, is an improvement over previous methods and is, in most respects, analogous to techniques developed for vertebrate cytopathic viruses.

MATERIALS AND METHODS

Cells and virus. The following cell lines were used: *Spodoptera frugiperda* (fall army worm) cell line IPLB-SF-21 (11), passages 185 to 220; *Trichoplusia ni* (TN-368, cabbage looper) cells (7) beyond passage 1,000; *Bombyx mori* (silkworm) cells (6), passages 30 to 50; and *Manduca sexta* (tobacco hornworm) cells (4). The passage level of the *M. sexta* cells is not known, but the cells had been carried in our laboratory for 45 passages by the time these experiments were completed.

All cell lines were cultivated at 27°C in BML-TC/10 medium (5) containing gentamicin (50 μg/ml).

The nuclear polyhedrosis virus (NPV) was grown from a plaque-purified stock of *Autographa californica* MNPV which expressed the MP phenotype (9, 10). It was grown in *S. frugiperda* cells, was used at the third passage level, and is referred to here as *A. californica* MP3 (SF). An FP strain of the virus (9, 10) was grown in TN-368 cells from a plaque-purified FP stock of *T. ni* NPV and was also used at the third passage level. Unless otherwise stated, the indicator cells used in the plaque assay were *S. frugiperda* and the virus inoculum was *A. californica* MP3 (SF).

Plaque assay procedure. Cells (2-ml suspension) were seeded in plastic tissue culture petri dishes (35-mm diameter; Corning) and were left to attach for 1 to 7 h at room temperature. Routinely, more than 90% of the cells were viable as determined by trypan blue exclusion. The medium was removed by suction, and 0.1 ml of virus inoculum was added to the center of each plate. Plates were rocked, in a humid container, on a Bellco rocker platform (1 oscillation per min) for 1 h at room temperature. The inoculum was withdrawn by suction, and 2 ml of overlay was added to each plate. Overlay medium contained Seaplaque or Seakem agarose (1.5%; Marine Colloids, Inc.) or 1.5% agarose (Miles Laboratories, Inc.) as the solidifying agent. In the experiments reported here, two formations of overlay medium were used. For overlay type A, a 3% solution of agarose was prepared in distilled water, autoclaved, cooled to 45°C, and mixed in equal proportions with BML-TC/10 containing 10% fetal calf serum preheated to 45°C. After the agarose overlay solidified, 1 ml of BML-TC/10 was added as a liquid overlay to each plate. For agarose overlay type B, 3% agarose was autoclaved in BML-TC/10 lacking fetal calf serum, cooled to 45°C, and mixed in equal proportions with BML-TC/10 containing 10% fetal calf serum preheated to 45°C. No liquid overlay was added. Plates were incubated in a humid container at 27°C for 3 to 6 days. After removal of the liquid overlay (if present), cells were stained with 1 ml of INT [0.2% (wt/vol) 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl-tetrazolium chloride in phosphate-buffered saline: 140 mM NaCl, 27 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4 (pH 7.3)] or with 1 ml of neutral red (0.1% [wt/vol] in phosphate-buffered saline) for 2 to 4 h at 27°C. The stain was then removed, and plaques were counted the following day.

Methyl cellulose (0.6%) was used as an overlay in some comparison experiments. The overlay, prepared as described by Hink and Vail (9), was made up in BML-TC/10. Plates were stained 4 days postinfection by addition of 1 ml of neutral red (0.1% [wt/vol] in phosphate-buffered saline) to each plate. The viscous overlay was poured off after 5 h at 27°C, and the

plaques were scored by microscopic examination of each entire plate.

End-point dilution titration. End-point dilution titration was done as previously described (1). A seeding concentration of 2,000 cells per well was used.

RESULTS

Optimal conditions for plaque assay using a solid overlay. We showed in an earlier report (2) that NPV may be titrated by plaque assay using an agarose overlay containing modified culture medium of low salt concentration (agarose overlay type A). Plaques were formed only when the solid overlay was covered with a layer of complete BML-TC/10. Subsequently, we modified the agarose overlay to type B and found that the liquid layer was no longer necessary for plaque formation (Table 1).

Microscopic examination of the plates before staining indicated that cells under the overlay to which double-strength culture medium had been added appeared to undergo osmotic shock and exhibited no growth. Cell growth was poor under the overlay containing agarose autoclaved in distilled water, and no plaques were observed. Plaques were formed, however, under the overlay containing Seakem agarose autoclaved in serum-free culture medium and mixed in equal proportions with BML-TC/10 medium containing 10% fetal calf serum (type B overlay). Seaplaque agarose and agarose also supported the formation of plaques under these conditions without the use of a liquid overlay. For overlay containing either Seaplaque agarose or agarose, the greatest number of distinct plaques appeared 4 days postinfection on plates seeded with 10^6 viable cells.

TABLE 1. *Development of plaques without BML-TC/10 layer*[a]

Composition of overlay	Visible plaques
Seakem agarose autoclaved in distilled water, mixed in equal proportions with BML-TC/10 medium containing 10% fetal calf serum	—
Seakem agarose autoclaved in distilled water, mixed in equal proportions with double-strength BML-TC/10 medium containing 10% fetal calf serum	—
Seakem agarose autoclaved in BML-TC/10 medium lacking serum, mixed in equal proportions with BML-TC/10 medium containing 10% fetal calf serum (type B overlay)	+
Seakem agarose autoclaved in BML-TC/10 medium lacking serum, mixed in equal proportions with double-strength BML-TC/10 medium containing 10% fetal calf serum	—

[a] Plastic tissue culture petri plates (35-mm diameter) were seeded with 10^6 viable cells and inoculated with a virus dilution known to yield a countable number of plaques. The overlay was prepared as described. Cells were stained with neutral red 6 days postinfection.

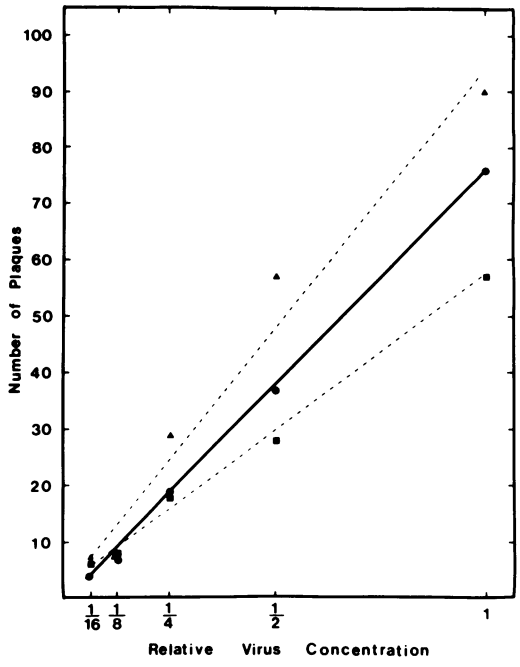

FIG. 1. *Dose-response relationship for comparison of type A and type B overlays. Three dose-response assays were set up to compare the sensitivities of plaque assay using type A and type B overlays. Plates were seeded with 10^6 cells, and duplicate plates were inoculated with twofold dilutions of A. californica MP3 (SF). Cells were stained with neutral red 4 days postinfection. Symbols: ▲, agarose, type A overlay; ●, agarose, type B overlay; ■, Seaplaque agarose, type B overlay.*

Dose-response experiments were set up to compare the sensitivities of assays using type A and B solid agarose overlays (Fig. 1). In each case, the number of plaques was proportional to the virus concentration. The sensitivity of a plaque assay can be determined by calculation of the ratio plaque-forming units (PFU) per 50% tissue culture infective dose ($TCID_{50}$) when both the plaque assay and end-point dilution are done with indicator cells from the same cell line. According to Poisson distribution, this ratio should be 0.7 (3). The $PFU/TCID_{50}$ ratios for several plaque assays of *A. californica* MP3 (SF) on *S. frugiperda* indicator cells under different overlay conditions are listed in Table 2. Although the ratio varied, the data indicate that the BML-TC/10 layer (type A overlay) was not required since $PFU/TCID_{50}$ ratios ranged from

0.47 to 0.66 for overlay type B containing agarose. Consequently, no liquid overlay was used in subsequent virus assays, with the advantages that less manipulation was involved, contamination was reduced, and plates could be inverted during incubation.

Comparison of solid agarose and methyl cellulose overlays. An experiment was set up to compare the sensitivities of assays using type B agarose and methyl cellulose overlays. The same *A. californica* MP3 (SF) preparation was titrated concurrently by both methods and gave titers of 6.0×10^6 and 3.2×10^6 PFU/ml for the agarose and methyl cellulose overlays, respectively.

Plaque morphology. Plaques formed under the solid agarose overlay and counted 4 days postinfection ranged in diameter from approximately 0.5 to 2 mm and were similar in appearance to those described previously (2). Plaque size was not under genetic control of the virus since progeny picked from well-isolated, distinct plaques gave rise to plaques of various diameters when replated.

Use of Seaplaque agarose with or without a liquid overlay sometimes resulted in the formation of elongated plaques (ca. 2 by 5 mm). Such plaques were not observed under the overlay containing either Seakem agarose or agarose, but the reason for this is unknown. The formation of such elongated plaques can obscure smaller plaques due to overlap, resulting in a decreased plaque count, as seen in Fig. 1.

Both MP and FP plaques could be seen macroscopically without staining but were easier to count after the monolayers were stained with neutral red or INT. Microscopic examination of the infected monolayers indicated that the plaques were foci of unstained cells, lighter in density than the surrounding area. Polyhedra were observed in cells at the center of the focus but not in the unstained cells at the periphery. Cell lysis was not usually observed at the focus of infection.

Plaques formed under the methyl cellulose overlay could be seen without the aid of a microscope after staining with neutral red but were very small (<0.5 mm). These plaques were marked and then examined under the microscope; they proved to be true foci of infection.

Use of the plaque assay with cells other than *S. frugiperda*. The plaque assay procedure using type A overlay was tried with TN-368 cells and with cells of the silkworm *B. mori*. Visible plaques had a clear center surrounded by a ring of cells which exhibited increased uptake of neutral red. However, plaque assays with TN-368 and *B. mori* cells were not reliable, since the appearance of visible plaques depended on the development of an even monolayer of cells in the plates, and this was not routinely possible. In one experiment, large clear plaques were formed on TN-368 cell layers but did not represent all the foci of infection present. The progeny of these large plaques bred true when replated on TN-368 cells but did not yield large plaques when plated on *S. frugiperda* cells.

Visible plaques were also formed on monolayers of tobacco hornworm, *M. sexta*, cells with type A overlay. Plaques were clear but not as distinct as those on *S. frugiperda* monolayers since uninfected *M. sexta* cells stain less intensely than do uninfected *S. frugiperda* cells. MP and FP plaques could be distinguished on *M. sexta*, but not on *S. frugiperda*, monolayers by microscopic examination. However, *M. sexta* cells are less sensitive to infection with NPV than are *S. frugiperda* cells. The same stock of *A. californica* MP3 (SF) was titrated by the end-point dilution method in TN-368 cells and by both plaque and end-point dilution methods in *S. frugiperda* and *M. sexta* cells. The results (Table 3) showed that these cell lines differed in sensitivity to infection with NPV. However, the PFU/$TCID_{50}$ ratios for assays in which *M. sexta* and *S. frugiperda* cells were used as indicators were comparable when the same cells were used for both the plaque assay and end-point dilution titrations. Since *M. sexta* cells were considerably less sensitive to infection with NPV than were *S. frugiperda* cells, they are not recommended for routine assays.

TABLE 2. *Sensitivity of the plaque assay under different overlay conditions*

Solidifying agent	Overlay type	PFU/$TCID_{50}$[a]
Seaplaque agarose	A	0.44
		0.55
		0.52
		0.30
Seaplaque agarose	B	0.38
Seakem agarose	A	0.58
		0.51
Agarose	A	0.65
		0.60
Agarose	B	0.47
		0.66
		0.52

[a] Ratios were calculated from data obtained in several plaque assay experiments done as described in the text. The same $TCID_{50}$ titer was determined in two separate end-point dilution titrations.

TABLE 3. *Titration of A. californica NPV in cell lines from three insects*[a]

Indicator cells	Overlay type	Titer		PFU/$TCID_{50}$
		$TCID_{50}$/ml	PFU/ml	
T. ni	ND[b]	2.3×10^8	ND	ND
S. frugiperda	B	6.3×10^7	3.0×10^7	0.47
			3.3×10^7	0.52
			4.2×10^7	0.66
M. sexta	A	8.7×10^4	5.7×10^4	0.66
			3.2×10^4	0.37

[a] The same stock of *A. californica* MP3 (SF) was titrated by the end-point dilution method in TN-368 cells and by both end-point dilution and plaque assay methods in *S. frugiperda* and *M. sexta* cells. For each cell line, the PFU/$TCID_{50}$ ratio was calculated from the titers obtained using that cell line.

[b] ND, Not done.

DISCUSSION

A plaque assay technique has been developed which has advantages over previously described methods. This technique employs a solid overlay and yields plaques which are easily counted 4 days postinfection without the aid of a microscope. Although the methyl cellulose overlay described by Hink and Vail (9) has recently been modified to increase plaque size and sensitivity (8), plaques are still counted by using a microscope. In addition, the length of incubation has been increased from 3 days to 6 or 7 days postinfection. PFU/$TCID_{50}$ ratios obtained with *A. californica* NPV on *S. frugiperda* cells, using the solid agarose overlay, ranged from 0.47 to 0.66 (Table 2). With a methyl cellulose overlay (9), Potter (Ph.D. thesis, Queen's University, Kingston, Ontario, Canada, 1977) obtained a PFU/$TCID_{50}$ ratio of 0.3 for both the MP and FP strains of *T. ni* NPV (10) on TN-368 cells. Comparative titers of the same *A. californica* MP3 (SF) preparation assayed on *S. frugiperda* cells with type B agarose and methyl cellulose overlays were 6.0×10^6 and 3.2×10^6 PFU/ml, respectively. This indicates that the sensitivity of the plaque assay was increased somewhat with the use of a solid agarose, rather than methyl cellulose, overlay. In addition, plaques formed under solid agarose are more easily visualized with or without staining than those formed under viscous methyl cellulose, thus permitting easier quantitation and cloning of the virus. Plaque formation under methyl cellulose overlay, followed by neutral red staining, is not feasible for cloning of virus for two reasons: (i) the overlay, and hence the released virus, must be removed to score the plaques; (ii) NPV is inactivated in the presence of neutral red (results not shown) and therefore should be cloned in the absence of stain. Both *A. californica* (9) and *T. ni* (10) NPVs have been cloned using a methyl cellulose overlay, but this involves tedious microscopic examination of the entire plate to locate the plaques.

Wood (12; Proc. First Int. Colloq. Invertebr. Pathol. and IXth Annu. Meet. Soc. Invertebr. Pathol., 1976, abstract, p. 401–402) described a plaque assay of *A. californica* NPV on TN-368 cells, using a solid overlay containing Seaplaque agarose, in which the virus was centrifuged onto the cells. He found it necessary to seed the cells in multiwell culture dishes since it was difficult to obtain even monolayers in larger dishes. This is consistent with observations reported above for TN-368 cells. The PFU/$TCID_{50}$ ratios calculated from the data presented by Wood (12) were 0.4 and 0.6 in two experiments. Comparable ratios were obtained without the necessity of centrifugation in the plaque assays reported here.

The plaque assay technique described in this report does not require modification of the culture medium, unlike the method described by Knudson (Proc. First Int. Colloq. Invertebr. Pathol. and IXth Annu. Meet. Soc. Invertebr. Pathol., 1976, abstract, p. 104–107). A modified culture medium was also used in the method described by these authors in a previous communication (2). The necessity of adding culture medium as a liquid overlay above the solid agarose (type A overlay) (2) can be eliminated by autoclaving the agarose in a culture medium that lacks serum rather than in distilled water (type B overlay). Although the nutritive value of the culture medium is destroyed by autoclaving, the osmotic pressure is maintained so that the cells do not undergo osmotic shock when overlaid with the agarose. This overlay was chosen as the one to be used for routine plaque assays since the plating efficiency is suitable (Table 2) and, since less manipulation is involved, contamination is reduced and the plates can be inverted during incubation.

Both *S. frugiperda* and *M. sexta* cells were used as indicator cells for the plaque assay, but *M. sexta* cells were much less sensitive to infection with NPV than were *S. frugiperda* cells (Table 3). Neither TN-368 nor *B. mori* cells were satisfactory as indicator cells for the plaque assay since they did not consistently form good monolayers.

Thus it is now possible to quantitate NPV by plaque assay using a solid overlay without expensive purified agarose or modified culture medium. Although this technique does not surpass the end-point dilution method for routine titration of virus preparations, it is valuable for the cloning of virus in genetic studies.

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